# Transforming growth factor-$\beta$ released by apoptotic white blood cells during red blood cell storage promotes transfusion-induced alloimmunomodulation.

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# TGF-β released by apoptotic white blood cells during red blood cell storage promotes transfusion-induced alloimmunomodulation

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**Abstract**

BACKGROUND: Red blood cell alloimmunization is a major immunological risk of transfusion. However, RBC storage facilitates WBC apoptosis and apoptotic cells have immunomodulatory properties. We investigated the behavior of WBCs, and apoptosis in particular, in RBC units during storage, and then studied the impact of WBC-apoptosis on the modulation of post-transfusion alloimmunization in RBC products stored short term. STUDY DESIGN AND METHODS: We used a mouse model of alloimmunization to transfused HOD surface antigen expressed specifically on RBCs. The presence of circulating anti-HOD IgG detected by FACS confirmed immunization to $HOD^+$ RBCs. WBC apoptosis and factors released by apoptotic WBC during storage were determined and in particular the role of TGF-β was assessed on RBC alloimmunization. RESULTS: In blood stored 72 hours, 30% of WBCs were apoptotic, and transfusion of short-term stored blood resulted in lesser immunization than did fresh blood or stored leukoreduced RBCs (LR RBCs). WBC undergoing apoptosis released during short term storage factors modulating RBC alloimmunization. Indeed apoptotic cell-released factors modulate alloimmunization whereas exogenous apoptotic cells directly transfused with LR RBC did not. While microparticles released during RBC storage had no immunomodulatory role, TGF-β found in the supernatant of stored blood demonstrated the capacity to favor Treg polarization of naïve $CD4^+CD25^-$ T cells *in vitro* and limited RBC alloimmunization *in vivo*. Indeed, addition of recombinant TGF-β to stored LR RBC transfusion strongly limited post-transfusion RBC alloimmunization. CONCLUSION: Our findings show that short-term storage of non-leukoreduced blood facilitates WBC apoptosis therefore releasing TGF-β that modulates post-transfusion RBC alloimmunization.

**Key words:** apoptotic WBC, RBC, alloimmunization, transfusion, TGF-β

## INTRODUCTION

Post-transfusion alloimmunization is the chief immune-mediated adverse effect of transfusion.[1] Alloantibodies to RBC antigens are responsible for transfusion-related hemolytic reactions with potentially lethal consequences, transfusion failure when compatible RBC units cannot be found, and hemolytic disease of the newborn in women who were immunized prior to pregnancy. Alloimmunization to RBC occurs in <10% of recipients and can be triggered by immunogenetic predispositions[2] and other factors such as the inflammatory status of the recipient at the time of transfusion. The number (<40) or the intensity of transfusions do not seem to increase the risk of RBC alloimmunization.[3] Storage of RBC units and leukoreduction may however, have a significant impact on RBC alloimmunization. Indeed, experimental rodent models have shown that immunogenicity of RBC units increases with storage time[4]. In Humans, blood storage has been associated with increased inflammatory cytokine levels (TNF and IL-6) and higher priming capacities of neutrophils (PMN).[5-7] Leukoreduction reduced PMN activation by RBC supernatants, activation which increases with prolonged storage,[7] suggesting that WBC make stored RBCs immunogenic. This has been indirectly addressed in humans in a study that investigated the rate of alloimmunization to RhD –the most immunogenic RBC antigen– during different storage lengths.[8] The authors were unable to associate anti-D alloimmunization with RBC unit storage duration.[8]

RBC integrity can also be affected during storage,[9-11] notably through membrane-encapsulated hemoglobin iron that promotes immunogenicity of RBCs as demonstrated in an experimental model.[12] Interestingly, supernatants from stored RBCs did not produce the same inflammatory signal *in vivo* as stored RBCs.[12] Of note, in that study, RBC units were leukoreduced.[12] WBCs may also dramatically affect RBC alloimmunization. Indeed, WBC apoptosis during storage has

been described[13] and apoptotic cells are known to have imunomodulatory properties.[14-16] Some authors have speculated that in addition to soluble factors,[17,18] apoptotic WBCs may be essential to transfusion-related immunomodulation.[19]

To address the role of WBCs in RBC alloimmunization, we first studied WBC behavior during storage in RBC units, notably apoptosis, and then the impact of apoptotic WBCs on RBC alloimmunization using an experimental mouse model of post-transfusion alloimmunization.

## MATERIALS AND METHODS

### Mice

C57Bl/6, BALB/c and FVB mice were purchased from Charles River laboratories (L'Arbresle, France). Membrane-bound hen egg lysozyme (mHEL) C57Bl/6 and HEL-ovalbumin-duffy (HOD) FvB transgenic mice were kindly provided by Dr J Zimring (Puget Sound Blood Center Research Institute, Seattle, WA, USA) and bred in our specific pathogen-free rodent facility (agreement #D25-056-7) with food and water available *ad libitum*. Experimentation (#10004R) was approved by the local ethics committee (#58, approved by the French Ministry of Higher Education and Research [*Ministère de l'Enseignement Supérieur et de la Recherche*]) and conducted in accordance with the European Union's Directive 2010/63.

### RBC preparation and transfusion

Blood was collected by retro orbital bleeding into ethylenediaminetetra acetic acid (EDTA) or acid citrate dextrose (ACD) tubes (BD Biosciences) and leukoreduced (=LR RBC) or washed 3 times (=blood) to reduce platelet count (15 min, 300 g): $628 \times 10^6 \pm 189 \times 10^6$ of $CD61^+$ platelets/mL in whole blood, $4.3 \times 10^6 \pm 0.4 \times 10^6$ platelets /mL in washed blood ($P<0.05$ *vs*

whole blood), 2.7 x $10^6$ ± 0.2 x $10^6$ platelets /mL ± in LR RBCs ($P$<0.05 *vs* whole blood; $P$<0.05 *vs* washed blood); n=3 independent experiments pooled together; Student's unpaired *t* test. ~~Washed blood was then leukoreduced (=RBCs) or not (=non-LR blood or blood)~~ Leukoreduction was performed using a RN1 filter (Haemonatics, Plaisir, France) and included 3 washes ~~times~~ to remove platelets and plasma and resuspended in PBS or saline-adenine-glucose-manitol (SAGM) solution~~, and used or stored (+4°C)~~. Leukoreduction was quantified by FACS (CantoII, BD Biosciences, Le Pont de Claix, France) using Trucount tubes (BD Biosciences), as previously described.[20] WBC counts were greatly decreased from 6.3 x $10^6$ ± 0.3 x $10^6$ to 7.0 x $10^3$ ± 4.5 x $10^3$ WBC/mL (n=4; $P$<0.001, Student's paired *t*-test). Blood or LR RBCs (100 µL) were adjusted for RBC number and volume and then transfused *via* the lateral tail vein (500 µL in PBS or SAGM). Hematocrits were controlled using micro hematocrit tubes (Brand GMBH, Wertheim, Germany). Mice received 100 µg of poly(I:C) ip (Amersham Biosciences, Piscataway, NJ, USA) diluted in PBS, 4 h before transfusion as priming, a prerequisite for high alloantibody titers. To mimic bedside leukoreduction, stored blood supernatant was collected before leukoreduction to re-suspend stored RBCs in their own supernatant. Apoptosis and membrane-bound TGF-β expression (using latency-associated peptide [LAP] antibody, clone TW7-16B4, BD Biosciences) were evaluated in RBC units by FACS using APC-conjugated $CD45_{LCA}$ antibody, PE-conjugated Annexin-V and 7-AAD (BD Biosciences) according to the manufacturer's procedure, on gated $CD45_{LCA}^+$ cells, $CD45_{LCA}^+GR1^+CD11b^+$ neutrophils, $CD45_{LCA}^+GR1^-CD11b^+$ monocytes/macrophages, $CD45_{LCA}^+CD19^+$ B cells and $CD45_{LCA}^+CD3^+$ T cells. $HOD^+$ RBC survival was assessed in peripheral blood at different time-points post-transfusion, by $HOD^+$ RBC detection by FACS cross-match using plasma from mice immunized to HEL protein, followed by APC-conjugated goat anti-mouse IgG Ab staining.

**Alloimmunization evaluation**

Circulating anti-HOD IgG levels were quantified by FACS in the plasma of mice two weeks after transfusion. Plasma was diluted 1:4 in PBS and incubated with $HOD^+$ RBCs for 30 min, then washed and incubated with conjugated secondary anti-mouse IgG APC (SouthernBiotech, Birmingham, Alabama, USA). Finally, APC-intensity was evaluated by FACS on gated RBCs confirming the presence of anti-HOD Ab. Mean fluorescence intensity (MFI) ratio (MFI R) corresponded to MFI from sample/mean MFI from controls (transfused with vehicle). Anti-HOD Ab-positive plasma from mice immunized with HEL and ovalbumin proteins in complete Freund's adjuvant were used as crossmatch positive controls.

**Induction of apoptotic leukocytes**

WBCs or thymic cells were subjected to a 35-Gray X-ray radiation (Raycell blood irradiator, Best Theratronic Ltd, Ottawa, Canada) and cultured 6 h in complete DMEM ($10^6$ cells/mL) to allow apoptosis.[21] Apoptosis was confirmed by FACS using Annexin-V staining (BD Biosciences) and 7-AAD exclusion. Cells were considered as apoptotic when positive for Annexin-V and negative for 7-AAD staining. Annexin-V and 7-AAD positive cells were considered as necrotic. Apoptotic cell used were early-stage apoptotic (70-85% Annexin-$V^+$7-$AAD^-$ cells and <10% 7-$AAD^+$ cells). Apoptotic cells were washed in PBS and injected (5.10e6 cells/mouse) with LR RBCs, stored for 72 h at 4°C and injected with LR RBCs, or stored for 72 h at 4°C to collect supernatant and inject it with LR RBCs.

**Cell cultures with RBC supernatants**

Spleen cells or 90% enriched $CD4^+CD25^-$ T cells (using Miltenyi Biotec isolation kit) were cultured ($10^6$ cells/mL) for 72 h in complete DMEM with soluble anti-CD3ε mAb (0.5 µg/mL; clone 145-2C11; eBioscience) or plate-bound anti-CD3ε mAb (5 µg/mL) and soluble anti-CD28

mAb (2 µg/mL; clone 37.51; eBioscience), respectively. Supernatant from fresh or stored LR RBCs or blood were added to the culture (20% final volume). Recombinant TGF-β1 was used as a Foxp3 induction-positive control (5 ng/mL; R&D Systems, Minneapolis, MN, USA). After culture, cells were stained for CD4 and CD25 molecules and intracellular Foxp3 transcription factor, according to the manufacturer's instructions (eBioscience), and analyzed by FACS.

**TGF-β measurement and blockage**

Total and/or latent forms of TGF-β were quantified in RBC unit supernatants by ELISA following the manufacturer's instructions (Promega, Madison, WI, USA; Biolegend, Ozyme, Saint-Quentin-en-Yvelines, France). Anti-TGF-β monoclonal mAb (clone 2G7) was used (50 µg/mL) to neutralize TGF-β in stored RBC 30 min before transfusion. TGF-β-depletion in RBC units was performed using anti-TGF-β (clone 2G7) mAb coated on goat anti-mouse IgG BioMag beads (50 µg of mAb + 0.5 mL beads for 1 mL of RBC unit) according to the manufacturer's instructions (Polysciences, Warrington, PA, USA).

**Statistics**

Group comparisons of continuous data were made using Student's *t*-test or the Mann-Whitney Rank Sum test. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad software, San Diego, CA, USA). *P*-values <0.05 were considered significant.

**RESULTS**

**WBC underwent apoptosis in RBC units upon storage**

Storage of RBC units triggers WBC apoptosis and/or necrosis.[13] WBC-behavior was evaluated post-puncture in blood stored at +4°C in PBS or SAGM for 14 days. Apoptosis of WBCs,

hematocrits, *in vivo* survival and ultimately alloimmunization were assessed at different time-points. WBC numbers decreased progressively from Day 0 to Day 14, when the number of WBCs remaining was less than $10^6$ cells/mL (Fig. 1A). This was around 20% of the initial number of WBCs in fresh blood. Soon after storage, WBCs began to undergo apoptosis and this proportion (around 20 to 30% of WBC) remained constant over the storage-time (Fig. 1A,B). In contrast, we observed that WBCs slowly underwent necrosis after storage but at around Day 6-8, necrotic cell death became more marked until Day 14 when necrotic WBCs represented approx. 60% of all WBCs (Fig. 1A). WBC apoptosis or necrosis was not influenced by the medium used for storage (PBS *vs* SAGM) (Fig. 1C), nor by the anticoagulant solution used (EDTA *vs* ACD) (Fig. 1D). Hematocrits were found to be slightly unchanged throughout storage time and independently of the anticoagulant solution used (Fig. 1E). After 3 days of storage, SAGM seemed to better protect RBCs from hemolysis; this difference was not observable at Day 14. Hemolysis was associated with our 3 washes step procedure (data not shown). Survival of RBCs was not found to be significantly different according to the storage medium used; although storage itself seemed to affect *in vivo* RBC survival (Fig. 1F). Since WBC apoptosis was not influenced by experimental settings at Day 3 of storage, we then addressed how WBC apoptosis influenced RBC alloimmunization.

**Presence of apoptotic WBCs influenced post-transfusion alloimmunization**

The presence of apoptotic WBCs in RBC units presumably decreases immunogenicity because apoptotic cells have immunomodulatory properties.[15,16] Therefore, in an experimental model of post-transfusion alloimmunization, we investigated whether the presence of apoptotic WBCs affects RBC alloimmunization. We compared transfusion of fresh blood with that of blood stored

for 72 h ~~since this time point presented the best apoptotic/necrotic cell ratio (Fig. 1A,B)~~ since at day 3 of storage, stored blood products exhibit apoptotic WBCs (Fig. 1A,B). RBC stored for 72 hours is roughly equivalent to storage of human RBCs for 6-7 days.[22] WBCs are extremely sensitive to cold temperatures and rapidly undergo apoptosis with DNA laddering during routine 72-h storage at 4°C.[23] We used blood from HOD transgenic mice –only RBCs express membrane-bound HOD– that was either leukoreduced, stored for 72 h, or both.[24] Post-transfusion alloimmunization was evaluated two weeks post-transfusion through the detection of anti-HOD alloantibodies. First, we observed that unprimed mice transfused with fresh blood demonstrated stronger alloimmunization than mice receiving fresh LR RBCs ($P<0.01$; Fig. 2A,B), and this was also manifest in poly(I:C)-primed transfused mice ($P<0.01$; Fig. 2C,D). This suggested that leukoreduction may have decreased immunogenicity of fresh RBCs by removing immunogenic WBCs. In contrast, when blood and LR RBCs were stored for 72 h before transfusion, leukoreduction appeared to promote alloimmunization since mice receiving short-term stored blood demonstrated a lower magnitude of immunization than those receiving stored LR RBCs (Fig. 2A,B). This observation was significant in primed mice ($P<0.001$; Fig. 2C,D) and demonstrated that reducing WBC numbers before short-term storage increases immunogenicity of stored RBCs. Our findings showed that after short-term storage, without WBCs, RBCs displayed enhanced immunogenicity whereas in the presence of WBCs, they became less immunogenic, possibly due to WBC apoptosis. In addition, LR RBC immunogenicity observed after storage might be related to altered RBC integrity due to leukoreduction. Because 72 h-stored blood contained apoptotic WBCs (Fig. 1A,B), we hypothesized that apoptotic WBCs may modulate stored-blood alloimmunization.

**Addition of exogenous apoptotic cells to leukoreduced-RBC transfusion did not modulate post-transfusion alloimmunization**

Apoptotic cells are endowed with immunomodulatory properties that can control humoral immune response[25-29] independently of their strain origin.[30,31] We investigated whether addition of apoptotic cells would limit RBC alloimmunization. Alloimmunization was evaluated 2 weeks after transfusion of HOD LR RBCs plus apoptotic cells from different donor origins (FvB, C57Bl/6 or BALB/c origin); we observed no modulation of alloimmunization when apoptotic cells were transfused concomitantly with LR RBCs (Fig. 3A). Alloimmunization was not ameliorated either when the number of apoptotic cells was adapted to the number of WBCs injected into 72 h-stored blood ($0.3 \times 10^6$ apoptotic WBCs/transfusion) (Fig. 3B) nor when the time of injection was delayed from the time of transfusion to facilitate elimination of apoptotic cells by macrophages (Fig. 3C). Indeed, macrophages are essential to apoptotic cell-induced immunomodulation[21,29,32,33] and RBCs may compete with apoptotic cells, limiting both apoptotic cell removal by macrophages, and apoptotic cell-induced immunomodulation. Using $mHEL^+$ apoptotic cells (Figure 3 D) or apoptotic WBCs, as compared to apoptotic thymocytes (Fig. 3E), post-transfusion alloimmunization was not alleviated either. We also injected apoptotic cells with fresh or stored LR RBCs or blood, with no impact on alloimmunization (Fig. 3F). These data suggest that injecting apoptotic cells at the time of transfusion may not modulate RBC alloimmunization.

**Stored blood contained soluble factors limiting post-RBC-transfusion alloimmunization**

We then investigated whether the modulation of alloimmunization observed after transfusion of stored blood was related to the secretion of immunomodulatory factors by apoptotic cells within

the blood unit rather than to the presence of apoptotic cells. Firstly, to rule out the role of WBCs at the time of transfusion, we performed post-storage leukoreduction to eliminate WBCs, including apoptotic WBCs but not WBC-released soluble factors. This mimics bedside leukoreduction. In this setting, we observed that post-storage leukoreduction did not inhibit modulation associated with short-term stored-blood transfusion (Fig. 4A). This highlights the immunomodulatory role of soluble factors released by WBCs during storage. To confirm this, we used the supernatant from 72 h-stored blood to modulate stored-LR RBC alloimmunization; addition of stored-blood supernatant modulated stored-LR RBC alloimmunization (Fig. 4B). This confirms that the factors released by WBCs during short-term storage modulate alloimmunization. Transfusion of stored blood that was washed to eliminate soluble factors thus restoring RBC alloimmunization was used as a control (Fig. 4B).

To reconcile our data showing that addition of apoptotic cells to RBC transfusion did not ameliorate alloimmunization, with our data showing the critical role of stored blood-derived soluble factors on the amelioration of alloimmunization, we then investigated whether incubation of apoptotic cells with LR RBCs for 72 h would limit stored-LR RBC alloimmunization. We observed that mice transfused with LR RBCs stored with apoptotic cells demonstrated less alloimmunization than mice transfused with stored LR RBCs (Fig. 4C). This demonstrates that soluble factors released by apoptotic cells during 3-day storage modulated alloimmunization. Control mice transfused with stored LR RBCs plus 72 h-stored apoptotic cell supernatant again demonstrated reduced levels of RBC alloimmunization (Fig. 4C). Lastly, adding washed stored apoptotic cells to stored LR RBC transfusion did not modulate alloimmunization (Fig. 4C). These data confirm that WBCs undergoing apoptosis release soluble factors that reduce RBC immunogenicity.

**Stored blood contained TGF-β**

WBC-derived products are known to modulate immunogenicity of stored RBCs;[13,34] we therefore investigated the role of microparticles in stored-blood supernatant. Microparticles isolated from stored blood did not decrease stored-LR RBC alloimmunization (Fig. 4D). However, addition of the stored-blood supernatant remaining fraction −without microparticles− decreased stored-LR RBC alloimmunization (Fig. 4D).

To determine which factors other than microparticles could be responsible for reducing alloimmunization, we studied anti-inflammatory cytokines in stored-blood supernatant using T-cell polarization assays. Spleen cells were cultured in the presence of supernatants from stored and fresh LR RBCs or blood, and whereas differences were minimal in terms of Th1- or Th17-commitment of T cells (data not shown), stored-blood supernatant induced significantly greater expression of the Foxp3 transcription factor than other supernatants (Fig. 5A). This increase was equivalent to that observed with recombinant TGF-β as a control (Fig. 5A), suggesting the presence of TGF-β in stored blood. The same assay was performed using naïve $CD4^{+}CD25^{-}$ T cells; surprisingly, we did not observe any significant increase in Foxp3 expression in T cells cultured with stored-blood supernatant (Fig. 5B). To reconcile our data, latent TGF-β, which can be activated by spleen cells but not by naïve $CD4^{+}$ T cells, was quantified in stored blood by ELISA. We only observed the latent form in the tested supernatants (Fig. 5C) and latent TGF-β levels were significantly high only in stored-blood supernatants (Fig. 5C). Likewise, addition of blocking anti-TGF-β mAb to stored-blood supernatant in spleen cell culture strongly inhibited Foxp3 expression induced by stored-blood supernatant (Fig. 5D). Similar results were obtained using stored-blood supernatant depleted for TGF-β using magnetic beads (Fig. 5D). Our findings

confirm the presence of latent TGF-β in stored blood, which may modulate RBC alloimmunization.

**WBCs undergoing apoptosis during RBC storage secreted TGF-β**

Since our blood products were reduced in platelets, TGF-β must have been secreted by WBCs. We measured expression of membrane-bound TGF-β on leukocytes and subsets from fresh or 3 day-stored blood. Whereas T and B cells did not increase membrane-bound TGF-β expression over time, monocyte/macrophage populations, and neutrophils demonstrated slight and strong increases in membrane-bound TGF-β expression, respectively (Fig. 6A,C). Cells undergoing apoptosis are known to release TGF-β;[14] we therefore investigated leukocyte apoptosis. Leukocytes and neutrophils in particular, demonstrated more apoptosis after storage (Fig. 6B,C). Apoptotic leukocytes were positive for membrane-bound TGF-β-expression and as expected, this effect was strongly increased after 72 hours' storage (Fig. 6D,E). Necrotic cells demonstrated non-specific positivity for LAP expression. Our findings show that stored blood contains TGF-β released by WBCs undergoing apoptosis, mostly by the myeloid lineages.

**TGF-β contained in stored blood limited stored-RBC alloimmunization**

Apoptotic WBCs in stored blood released TGF-β, which may limit post-transfusion alloimmunization. Thus, TGF-β was neutralized using a blocking antibody in stored blood before transfusion and abrogated the modulation of stored-blood alloimmunization (Fig. 7A). However, residual anti-TGF-β antibody was also transfused. To avoid such a bias, we depleted TGF-β from stored blood using magnetic beads, thus achieving TGF-β-specific deletion before transfusion. In this setting, TGF-β-depleted stored blood no longer modulated alloimmunization (Fig. 7B). To

further confirm the key role of TGF-β, we first increased apoptotic cell-derived TGF-β content in stored apoptotic cell supernatants by storing 1, 5 or $15.10^6$ apoptotic cells per 400 µL of PBS that were then added (after cell elimination) to 100 µL of stored LR RBCs, as done in Figure 4C. Indeed, TGF-β content was increased in such supernatants from 53 ± 21 pg/mL for $1.10^6$ apoptotic cells to 709 ± 8 pg/mL for $15.10^6$ apoptotic cells. When those supernatants were transfused with stored LR RBCs, post-transfusion RBC alloimmunization was strongly reduced with $5.10^6$ apoptotic cell supernatant, and was almost prevented with $15.10^6$ apoptotic cell supernatant (Fig. 7C). Then we used recombinant TGF-β in quantities similar to those quantified in stored apoptotic cell supernatants, to limit stored LR RBC-induced alloimmunization. Interestingly, recombinant TGF-β was also able to decrease post-transfusion alloimmunization when used at 500 pg/mL of transfused blood, and to prevent alloimmunization when used at 1500 pg/mL (Fig. 7D). Our data indicate that apoptotic WBC-derived TGF-β can modulate post-transfusion alloimmunization and even prevent it when used at 1500 pg/mL.

Likewise, since Day 3 of storage was selected for the best apoptotic/necrotic cell ratio in RBC units, we observed that stored blood induced less immunization than stored LR RBCs only when storage lasted three days, and not six, eight or 14 days (Fig. 7E). Membrane-bound TGF-$\beta^+$ (=$LAP^+$) apoptotic WBC levels in blood were also seen to be higher after three days *versus* longer periods of storage, but no differences in terms of RBC alloimmunization modulation was observed after 3 days of storage (Fig. 7F). TGF-β levels in stored blood showed that low levels of latent TGF-β were present in LR RBCs (Fig. 7G). Whereas 3-day stored blood demonstrated high levels of latent TGF-β, significant amounts were also observed in blood stored longer (Fig. 7G), but with no effect on modulation of RBC alloimmunization (Fig. 7E,F), suggesting that other factors, possibly related to WBC necrosis, contributed to the higher immunogenicity of

RBCs stored long term. Our data highlight the key role of 3-day stored apoptotic WBC-derived TGF-β to limit post-transfusion RBC alloimmunization.

## DISCUSSION

In this study, we describe how WBCs that undergo apoptosis during storage, and more specifically apoptotic WBC-derived TGF-β, are associated with modulation of alloimmunization following transfusion of stored RBC. This is the first time that an experimental model of transfusion-induced alloimmunization has shown that apoptotic WBC-derived soluble factors induced by short-term blood storage, and in particular TGF-β, are responsible for immunomodulation of RBC immunogenicity. Fresh blood, LR RBCs and stored LR RBCs did not demonstrate such properties. Apoptotic WBC-derived soluble factors can negatively modulate transfusion-related alloimmunization. These data also highlight the crucial role of apoptotic WBCs in modulating immunogenicity of blood products thus confirming the role of TGF-β derived from apoptotic WBCs in the immunomodulation associated with transfusion ~~TRIM effect~~.

Viable leukocytes in transfused blood products have already been shown to be deleterious for recipients, especially for the incidence of alloimmunization.[35,36] Indeed, in our study, fresh LR RBC was less immunogenic than fresh blood, reinforcing the idea that WBCs promote ~~both~~ class I MHC immunization as well as RBC antigen immunization. However, after a short storage of 72 h, corresponding to approximately 6-7 days' storage in human settings, the presence of WBCs in stored blood modulated RBC immunogenicity. Our data suggest that with respect to minimizing post-transfusion alloimmunization, short-term blood storage might be beneficial ~~when short-term blood storage is being considered, leukoreduction should be done at the bedside and not before~~

~~storage~~. Firstly, blood preparation procedures might alter RBC integrity and LR RBCs became more immunogenic with time of storage. Secondly, when WBCs were kept in blood units, they become apoptotic and released TGF-β modulating stored-blood immunogenicity whereas after long period of storage, WBCs become necrotic and source of immunogeneic signals. Loss of RBC integrity was not address in this study. We used the now-well-established HOD mouse model to address the role of apoptotic WBCs on erythrocyte allo-immunization. For this purpose, we washed blood in order to avoid the influence of platelets. This step, not performed in human blood processing, influenced RBC integrity (data not shown) but similarly in all of our conditions, without affecting HOD expression on RBCs.

Our data highlight the crucial role of apoptotic WBC-derived immunomodulatory factors. Indeed, whereas injection of apoptotic cells has been shown to induce immunomodulation in inflammatory settings,[15,16] in our model of post-transfusion alloimmunization, it had no impact. ~~We expected the apoptotic cell-mediated effect to be limited by RBCs because once injected, RBCs would saturate macrophages thus eliminating apoptotic cells and mediating immunomodulation less efficiently.~~ Apoptotic cells were injected at different time points from transfusion but with no benefit on RBC alloimmunization. However, when apoptotic cells were stored with RBCs, the former had time to release TGF-β into the RBC unit thus reducing RBC immunogenicity. A similar effect was mimicked using the supernatant from stored apoptotic cells transfused with LR RBCs. TGF-β was shown to be the key cytokine in this effect.

Prolonged storage increases immunogenicity of RBCs. This increase can be associated with LR affecting RBC integrity and/or with significantly greater WBC necrosis. The high rate of WBC necrosis in our setting can be related to our blood preparation procedure since 3 washes step facilitated cell necrosis particularly after 6 days of storage (data not shown). Necrotic WBCs

stimulate inflammation,[37] and several studies have highlighted the harmful effects of prolonged storage on post-transfusion alloimmunization.[12,38] We elected to use blood stored 72 hours and studied WBC apoptosis-related but not necrosis-related effects that are easier to assess with blood stored 14 days.

Apoptosis of WBCs during storage is well known,[23,39] and may explain the anti-inflammatory effect of blood transfusion.[19,34] Our result supports this hypothesis since the presence alone of apoptotic WBCs within blood modulates RBC alloimmunization. Apoptotic WBCs have to age within RBCs to release anti-inflammatory cytokines such as TGF-β. Indeed, addition of apoptotic leukocytes to LR RBCs at the time of transfusion did not limit post-transfusion alloimmunization whereas addition of stored apoptotic cell supernatant did. Thus, TGF-β, released by apoptotic WBCs,[14] is responsible for modulating RBC alloimmunization. The role of TGF-β in this setting was further confirmed by depleting TGF-β from stored blood, which reestablished immunogenicity to stored RBCs. Furthermore, addition of recombinant TGF-β (1500 pg/mL) to stored LR RBC transfusion can prevent post-transfusion alloimmunization in almost all mice. TGF-β is a key cytokine for $Foxp3^+$ Treg induction, both in humans and rodents.[40,41] Treg are known to suppress T-cell activation and initiation of the immune response. Recently, Treg have also been shown to directly abrogate humoral responses by inhibiting both B cell Ig-production and class switching,[42] killing B cells,[43] and preventing long-lived plasma cell commitment.[44] Moreover, one study reported that Treg injection prior to transfusion totally suppresses alloimmunization.[45] In our study, whereas stored-blood supernatant triggered Treg polarization *in vitro*, no differences in terms of Treg percentages or numbers at one, two or 14 days post-transfusion were observed (data not shown). The mechanisms behind alloimmunization modulation by stored WBC-derived TGF-β have yet to be elucidated. ~~We can~~

~~hypothesize that TGF-β directly affects and limits APC to create an efficient humoral response to RBC antigens.~~ Our data suggested that TGF-β modulated APC engulfment of RBCs Ag, processing and presentation that limited immune response to the RBC's Ag.

Finally, this is the first demonstration of the relation between storage-induced apoptosis of WBCs and modulation of transfusion immunogenicity. Our study highlights the ~~tolerogenic~~ ~~role~~ immunomodulatory properties of apoptotic WBCs and apoptotic WBC-derived TGF-β in modulating post-transfusion alloimmunization. Apoptotic WBC-derived TGF-β represents only the tip of the iceberg and many other environmental factors, including the inflammatory status of the recipient, may influence post-transfusion alloimmunization and other adverse effects of human transfusion. In such settings, however, the presence of apoptotic WBCs in blood after short-term storage limits RBC immunogenicity. ~~Human transfusion procedures should include LR at patients' bedside for blood units stored short term (6–7 days) and include LR for blood units selected for long-term storage~~.

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**Figure Legends**

**Figure 1. WBCs undergo apoptosis during blood storage independently of experimental settings, not influencing RBC hematocrits or RBC *in vivo* survival.** WBC numbers (white circles), apoptosis (grey bars) and necrosis (black bars) were evaluated in blood from HOD mice stored at +4°C on different days as indicated (**A**). Representative data pooled from two out of six independent experiments. Percent of WBC apoptosis was compared between Day 0 and Day 3 of storage (**B**). Data pooled from 16 independent experiments. ***= $P<0.001$ (Student's *t* test). Blood from HOD mice was stored at +4°C in PBS (open symbols) or SAGM (black symbols) and apoptotic (squares), necrotic (triangles) and live (circles) WBCs were evaluated at different days as indicated (**C**). The same experiment was done to assess the influence of the anticoagulant used for blood puncture, EDTA (open symbols) or ACD (black symbols) (**D**). Data from one of two representative experiments. Hematocrits were evaluated in RBC units upon storage in SAGM (black symbols) or PBS (open symbols), punctured using EDTA- (circles) or ACD-coated tubes (squares) (**E**). Data from one of three representative experiments. Survival of RBCs (squares) or LR RBCs (circles), fresh (open symbols) or after 72-h storage (black symbols) in SAGM (left panel) or PBS (right panel) was evaluated *in vivo* after transfusion by the detection of HOD+ RBCs within total RBCs in transfused wild type mice (**F**). Data from one of two representative experiments showing mean $\pm$ SEM, five mice/group.

**Figure 2. RBC alloimmunization is influenced by both leucoreduction and storage**. Post-transfusion alloimmunization was demonstrated by anti-HOD IgG antibody detection by cross-match in the plasma of unprimed (**A**,**B**) or poly(I:C) primed (**C**,**D**) mice two weeks after receiving vehicle, fresh or 72-h stored LR RBCs or blood. Data were expressed as MFI ratio (to

vehicle) (**A**,**C**) or MFI (**B**,**D**). Im. HEL group corresponded to plasma from mice immunized against HEL Ag. Representative histograms from flow cytometry cross-match analysis showing mouse serum are given in **B** and **D**: light grey=mice receiving vehicle, dark grey=mice transfused with the indicated RBC. Numbers given correspond to MFI of mice transfused with RBC units as indicated. Data from three to 11 independent experiments, 15 to 55 mice/group, showing mice individually and mean ± SEM. *= $P<0.05$; **= $P<0.01$; ***= $P<0.001$ (Student's *t* test).

**Figure 3. Apoptotic cell injection with leukoreduced RBC transfusion does not modulate post-transfusion alloimmunization**. Alloimmunization (*i.e.*, presence of circulating anti-HOD IgG) was evaluated by cross-match in the plasma of primed C57Bl/6 mice, two weeks after receiving leukoreduced HOD RBCs (LR RBCs) with PBS or 5 x $10^6$ apoptotic cells of FvB (FvB Apo) or C57Bl/6 (C57Bl/6 Apo) or BALB/c (BALB/c Apo) origin (**A**). Data from five independent experiments, 6 to 25 mice/group, showing mice individually and mean ± SEM. Apoptotic leukocytes (Apo) were also injected in different quantities: 0.1, 1 and 5 x $10^6$ cells/mouse and post-transfusion alloimmunization evaluated (**B**). Data from two independent experiments, 4 to 8 mice/group, showing mice individually and mean ± SEM. Apoptotic cell injection was also delayed (-2 and +2 h) or not, from LR RBC transfusion and post-transfusion alloimmunization evaluated (**C**). Data from two to four independent experiments, 8 to 19 mice/group, showing mice individually and mean ± SEM. Primed mice were transfused with $HOD^+$ LR RBCs, with or without 5 x $10^6$ apoptotic cells from mHEL transgenic mice (mHEL Apo). One group of mice received apoptotic cells alone. Post-transfusion alloimmunization was evaluated by cross-match and confirmed by the presence of Ab directed against HOD antigen

(**D**). Data from one experiment showing mice individually and mean ± SEM, 2 to 5 mice/group. *=$P<0.05$; ***=$P<0.001$ (Student's *t* test). Apoptotic cells prepared from thymus (thymo. Apo) or WBCs (WBC Apo) were compared to modulate alloimmunization when injected with LR RBCs (**E**). Data from one experiment showing mice individually and mean ± SEM, 2 to 5 mice/group. Apoptotic cells were also injected in addition to fresh or stored LR RBCs or blood (+Apo = black squares) and RBC alloimmunization evaluated (**F**). Data from one experiment, 2 to 5 mice/group, showing mice individually and mean ± SEM. *= $P<0.05$ (Student's *t* test). Im. HEL=control plasma from mice immunized against HEL Ag.

**Figure 4. Stored blood contains soluble factors limiting post-transfusion RBC alloimmunization**. Post-transfusion alloimmunization (*i.e.*, presence of circulating anti-HOD IgG) was evaluated by cross-match of the plasma of primed mice, two weeks after receiving fresh or stored, leukoreduced (LR) RBCs or blood, or stored blood subjected to LR after storage (stored blood + LR) (**A**). Data from two independent experiments showing individual mouse and mean ± SEM, 4 to 10 mice/group. In addition to stored LR RBCs or stored blood, other mice were transfused with stored blood that was washed to eliminate soluble factors, or stored LR RBCs plus stored-blood supernatant (sup) (**B**). Alloimmunization was determined as previously described. Data from three independent experiments showing mice individually and mean ± SEM, 8 to 15 mice/group. Mice were transfused with stored LR RBCs or blood, or LR RBCs stored with apoptotic cells (stored [LR RBCs+Apo]), stored LR RBCs plus stored apoptotic cells (+ stored Apo) or stored LR RBCs + the supernatant from stored apoptotic cells (+ stored Apo sup) (**C**). Alloimmunization was determined as previously described. Data from two independent experiments showing mice individually and mean ± SEM, 4 to 10 mice/group. To evaluate the

role of microparticles in RBC units, mice were transfused with stored LR RBCs or blood, stored blood washed to eliminate soluble factors, or stored LR RBCs plus stored-blood supernatant (+ stored blood sup), stored-blood microparticles (+ stored blood µP), stored-blood supernatant exempt of microparticles (+ stored blood sup wo µP) or both of the latter (+ stored blood µP + stored blood sup wo µP) (**D**). Alloimmunization was determined as described previously. Data from one experiment showing mice individually and mean ± SEM, five mice/group. *=$P$<0.05; **=$P$<0.01; ***=$P$<0.001 (Student's $t$ test). Im. HEL=control plasma from mice immunized against HEL Ag.

**Figure 5. Stored-blood supernatant contains TGF-β.** Spleen (**A**) or $CD4^+CD25^-$ (**B**) cells were cultured for three days in the presence or not (open bars) of CD3- or CD3/CD28-specific Abs (black bars), respectively, and with fresh or stored LR RBCs or blood supernatants. Polarization toward a regulatory T-cell profile was evaluated by FACS in $CD4^+$ T cells by measuring the expression of CD25 molecule and Foxp3 transcription factor. Data showing three pooled independent experiments mean ± SEM. Total (black bars) and active (open bars) forms of TGF-β were quantified by ELISA in corresponding supernatants (**C**). Mean ± SEM of duplicates from one of two representative experiments. Spleen cells were cultured as indicated in (**A**) in the presence of stored-blood supernatant and with or without (–) anti-TGF-β blocking antibody (+ αTGF-β), its corresponding isotype (+ iso) or with stored-blood supernatant depleted of TGF-β (TGF-β-depleted) (**D)**. Polarization toward a regulatory T-cell profile was evaluated similarly. Data from two pooled experiments, showing means of triplicates ± SEM. *=$P$<0.05; **=$P$<0.01; ***=$P$<0.001 (Student's $t$ test).

**Figure 6. WBCs express membrane-bound TGF-β on apoptosis during RBC storage**

Monocytes/macrophages ($CD11b^+$), neutrophils ($GR1^+$), B ($CD19^+$) and T ($CD3^+$) cells were gated in $CD45_{LCA}^+$ leukocytes for their expression of membrane-bound TGF-β (= LAP expression) (**A**,**C**) or apoptosis (**B**,**C**) by FACS during storage on leukocytes (diamonds), mono/macrophages (circles), neutrophils (triangles), T (crosses) and B (squares) cells in RBC units during storage as indicated. Leucocytes were also examined for the expression of membrane-bound TGF-β (= LAP expression) by FACS during storage of blood depending on their survival status (alive [7-$AAD^-$Annexin $V^-$], triangles; apoptotic [7-$AAD^-$Annexin $V^+$], circles; necrotic [7-$AAD^+$Annexin $V^+$], squares) (**D**,**E**). Percentages of cells of interest are shown on dot plots. Representative dot plots from one of three independent experiments showing similar results (**A**,**B**,**D**). Data from three pooled experiments showing mean ± SEM (**C**,**E**).

**Figure 7. TGF-β contained in stored blood modulates stored RBC alloimmunization.** Post-transfusion alloimmunization (*i.e.*, presence of circulating anti-HOD IgG) was evaluated by cross-match in the plasma of primed mice, two weeks after receiving fresh or stored LR RBCs or blood. In addition, some mice also received stored blood in which TGF-β had been neutralized prior to infusion using anti-TGF-β Ab (+ αTGF-β), or stored blood plus corresponding isotype (+ iso.) (**A**), or stored blood depleted from TGF-β using microbeads (stored blood TGF-β depleted) (**B**). Data from one to three independent experiments showing mice individually and mean ± SEM, five to 15 mice/group. Stored blood or LR RBC were transfused alone and stored LR RBCs were also transfused with apoptotic cell-derived TGF-β (+stored $1.10^6$ Apo sup., +stored $5.10^6$ Apo sup., +stored $1.10^6$ Apo sup.) (**C**) or with recombinant TGF-β (150, 500 and 1500 pg/mL of transfused blood) (**E**) and alloimmunization was detected in plasma two weeks

after transfusion. Data from two experiments showing mice individually and mean ± SEM, 4 to 10 mice/group. Blood or LR RBCs were transfused into mice either fresh (0 days) or after three, six, eight or 14 days' storage, and alloimmunization was detected in plasma (**E**). Data from one experiment showing mice individually and mean ± SEM, five mice/group. Alloimmunization to LR RBCs or blood was evaluated according to the duration of storage or the number of $LAP^+$ WBCs in blood (**F**). Data from one of two independent experiments showing mean ± SEM, five mice/group. Latent TGF-β was quantified by ELISA in blood and LR RBCs, fresh or stored as indicated (**G**). Data from one of two representative experiments showing mean of duplicates ± SEM. *=$P$<0.05; **=$P$<0.01; ***=$P$<0.001 (Student's $t$ test). Im. HEL=control plasma from mice immunized against HEL Ag.